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Canadian Science Advisory Secretariat
National Capital Region
Science Response 2014/042

ADVICE RELATED TO THE SAMPLING OF WILD FISH CARCASSES FOR THE PROPOSED *AQUACULTURE ACTIVITIES REGULATIONS* AQUACULTURE MONITORING STANDARD

Context

Fisheries and Oceans Canada's Aquaculture Management Directorate (AMD) is developing the *Aquaculture Activities Regulations* (AAR) under section 35 (fisheries protection) and section 36 (deposit of deleterious substances) of the *Fisheries Act* to manage potential impacts to fisheries and fisheries habitat resulting from aquaculture activities (i.e., the deposit of deleterious substances including drugs, pathogen and pest treatment products and the deposit of Biochemical Oxygen Demanding (BOD) matter).

The AAR Aquaculture Monitoring Standard (AMS) is being developed by AMD to support the implementation of the AAR. The Standard will be incorporated by reference and the procedures and methods contained within will be enforceable. Typically, the AMS will be used by aquaculture licence holders, their employees, and agents throughout Canada to meet monitoring requirements related to the deposit of deleterious substances as set out in the AAR.

AMD is requesting science advice to provide direction on the protocols to be followed when collecting carcasses of fish for analyses for chemotherapeutants following an unusual mortality or morbidity event. A comprehensive science review of the AAR or the associated Standard was not requested and is not within the scope of this current advisory response.

This Science Response Report results from the Science Response Process of July 10, 2014 on the Provision of advice related to the sampling of fish carcasses for the *Aquaculture Activities Regulations* Aquaculture Monitoring Standard.

Background

The proposed *Aquaculture Activities Regulations* (AAR) establish the conditions under which the owner or operator of an aquaculture facility may deposit drugs or pest control products. One of these conditions stipulates that if unusual fish morbidity or mortality outside the aquaculture facility is observed within 96 hours of a deposit of any drug or pest control product then the owner or operator must obtain tissue samples of the affected fish in accordance with the methodology specified in the Aquaculture Monitoring Standard (AMS).

The AMS will support the implementation of the environmental monitoring and sampling conditions set forth in the proposed AAR. It will be incorporated by reference and the procedures and methods contained within will be enforceable.

The Aquaculture Management Directorate (AMD) has requested science advice to inform development of the AMS regarding sampling protocols and considerations for chemotherapeutant analysis to support the aforementioned condition in the AAR on the deposit of drugs or pest control products. The National Contaminants Advisory Group (NCAG) provided the lead in developing this science advice.

Specifically, information provided is response to the following request for advice:

What are the procedures to be followed for the collection of wild fish carcasses for chemical analyses of the active ingredients found in anti-sea lice chemotherapeutant products? The recommended procedures should, at a minimum, address how aquaculture staff should collect the morbid or dead fish, where applicable, the number of fish to be collected, handling and preservation of the collected samples, timeframes for collection and preservation and how to ship the samples for analysis. Advice is also requested on limitations associated with the collection of fish samples for analysis considering the analytical request could target active ingredients from any and all anti-sea lice chemotherapeutants currently used in Canada.

These protocols are currently not readily available and there is a requirement to develop a specific protocol for the collection of these tissue samples within the context of the AAR requirements. In responding to this request, it is understood that the monitoring and sampling protocols outlined in the AMS will be used by aquaculture license holders, their employees, and their agents throughout Canada to meet the requirements related to the deposit of deleterious substances set out in the proposed *Aquaculture Activities Regulations* (see Section 34 of the *Fisheries Act* for the definition of deleterious substance).

DFO Science has provided AMD with previous CSAS advice on the potential exposure and associated biological effects from aquaculture pest and pathogen treatments (DFO 2013a, DFO 2013b). This advice characterized the lethal and sub-lethal toxic effects of four anti-sea lice pesticides on key indigenous non-target organisms (invertebrates) using laboratory toxicity testing and assessed the potential for indigenous non-target organisms in the environment to be exposed to biologically relevant concentrations of anti-sea lice pesticides post-treatment. In general, the effects on non-target organisms varied with the formulation being applied with lobster being the most sensitive species tested.

Analysis and Response

Which fish tissue samples should be collected at the aquaculture site for chemotherapeutants analysis?

Advice on the collection of samples has been provided here with the understanding samples would be collected by the aquaculture license holder, its employees or their agents.

For consistency and to maintain sample integrity, fish dissection and preparation of specific tissues for analysis should take place at the chosen analytical lab and should follow a standardized method based on species, life stage and analytical requirements. The specific tissue requirements including the type of tissue, the amount of tissue and required handling measures would be guided by the properties of the specific chemotherapeutants and the specific method of analysis to be employed.

Rather than have aquaculturists dissect fish and collect tissue samples, it is recommended that whole fish be collected and frozen with as little handling as possible. Aquaculture facilities are a source of contamination where treatment takes place or where therapeutants are stored, so sampling must be designed to reduce potential cross-contamination. Relevant expertise and a 'clean' environment are required for tissue collection; samples could be compromised where this is lacking.

Recommendation on tissue sampling and preparation

In summary, to avoid contamination and to ensure a consistent and standardized approach, it is recommended that whole fish be collected, frozen on-site and shipped to a lab for processing and analysis.

How many fish should be collected?

Of the three chemotherapeutants considered here, only emamectin and related chemicals are sufficiently persistent, with half-lives of the order of weeks (Horsberg 2012), to justify residue analysis which could relate mortality to the presence of the chemotherapeutant. However, to relate mortality in a putatively affected sample to drug exposure would require, at least, a parallel sampling and analysis of "reference" fish known *not* to have been exposed to emamectin. To calculate the minimum number of samples needed to establish convincingly a relationship between residue distribution and mortality requires information about (i) the mean residue concentrations and (ii) their standard deviation (SD) in both "affected" and "reference" groups, (iii) the statistical power desired (usually chosen arbitrarily to be 0.8) and (iv) the type 1 error rate (usually chosen arbitrarily to be 0.05). *Note that variables (i) and (ii) cannot usually be predicted in advance but will have to be determined empirically during or after an event.* However, the data of Glover et al. (2010) who measured emamectin residue concentrations in Atlantic Salmon following an intraperitoneal (IP) injection, at a dose rate roughly equivalent to the therapeutic dosage, provide a guide to estimating approximately the number of samples required. Appendix 1 shows in detail the calculations of the number(s) of samples required using the statistical approach of Rosner (2010) and based on the mean emamectin residue concentrations and variance reported by Glover et al. (2010). Assuming the investigator wants to detect a 5-fold difference between affected and reference group means, and fixing statistical power at 0.8 and type 1 error rate at 0.05, if the variance is 50% of the mean values, 5 samples would be required from each group, but if variance was, e.g., 100% of the mean, 13 samples would be required from each group; at a variance of 200% of mean, 51 samples per group are required (Figure 1). A similar effect is seen if the sample number (per group) is calculated for a range of differences in group means, again fixing power at 0.8, type 1 error rate to 0.05 and now fixing pooled SD at 100% of means. If instead of seeking a 5-fold difference between means of affected and reference group samples, the investigator seeks only a 2-fold difference, the sample size becomes 40 samples/group.

The data of Glover et al. (2010) were derived from a controlled experimental study in a laboratory, and in the field, variance will almost certainly be larger. Furthermore, the therapeutic dose of emamectin administered in food is about 14% of its toxic dose to Atlantic Salmon (Bright and Dionne 2005) so a kill resulting from an accidental overdose or release suggests that relatively large amounts of emamectin may be involved, leading to even wider variance in residue concentrations. The toxic dose varies amongst species. The data summarized in Appendix 1 show that if the variance is 100% of mean values, 13 samples per group would be required to detect a 5-fold difference between group means. If the variance were higher, or the expected difference between affected and reference groups were < 5-fold, considerably more samples would be required.

Samples from affected fish need to reflect the size, age and species distribution of the affected fish. The reference sample group should be collected so that the fish are of similar species and size, etc., as the affected group.

Reference samples should be collected as close in time as possible, but at a distance from where the unusual morbidity or mortality event occurred.

Recommendation on the number of fish to be sampled

A reasonable practical recommendation would therefore be *to sample and store for analysis around 50 fish each from affected and reference groups for each affected species, and to analyze initially 15 from each of the affected and reference groups to establish mean values and variances*; at this point a decision can be made as to whether more analyses are required to refine the statistical analyses.

Samples from affected fish need to reflect the size and species distribution of the affected fish. The reference sample group should be collected so that the fish are of similar species and sizes as the affected group.

What are the preferred methods for collecting, preserving and transporting fish samples from an aquaculture site to a lab?

The current AAR requires that when unusual fish morbidity or mortality (as per the *Fisheries Act* definition of fish) is viewed from any part of the aquaculture facility, within 96 hours after the deposit of any drug or pesticide product, the moribund and dead fish be collected. As discussed in '*Limitations and Considerations*', there are practical sampling limitations in addressing the full scope of this definition, thus sampling advice will be provided for finfish, bivalves and crustaceans.

For the sampling procedures described here, all recognized safety procedures, material handling requirements, etc. should be followed. The following sections provide recommendations on sample collection, preservation and shipping; maintenance of sample integrity; field sampling requirements; and suggested sampling supplies.

Sample Collection, Preservation and Shipping

Collection

1. In preparation for sampling, ensure that freezers and equipment are cleaned between sampling sessions using a residue-free lab detergent and are triple-rinsed with distilled or tap water.
2. Immediately prior to any chemotherapeutant treatment, prepare all required sampling supplies and equipment as described in Section *Suggested Sampling Equipment and Supplies* and transfer to the collection boat which should be ready for a rapid deployment.
3. Upon observation of unusual fish morbidity or mortality of fish of any species or life stage, site staff should immediately deploy the collection boat to locate the fish and begin sampling.
4. Throughout the collection process, field notes should be logged on standardized recording forms to document the field data variables described below in Section *Field Data Requirements*.
5. Video record behaviour of moribund fish where possible and include the sample identification label in the images.
6. Using a dip net, collect the fish from the water and immediately dispatch any live finfish using a clean wooden bat or equivalent device. The only exception would be that crustaceans should be chilled on ice until they are sufficiently stunned. Further, care should be taken to minimise exposure to freshwater during chilling and shipping.

7. Using sterile nitrile gloves, set the fish on a clean piece of aluminum foil to record the observations described below in Section *Field Data Requirements*.
8. Protect the integrity of the fish by ensuring no damage occurs to the outside (skin shell or carapace) or to the gills of the fish. External damage provides entry points for contamination and can lead to fluid loss which introduces variability in analytical results.
9. Record digital images of all fish and, ensuring that any morphological abnormalities and parasites are observed and that the sample identification label is included in the images.
10. Affected fish of the same species and of representative size should be collected, up to a maximum of 50 fish per species. See Section *How many fish should be collected?* for discussion on analytical considerations and recommendations for the collection of reference fish.
11. Where permissible, reference fish of similar size and species should be collected in the same numbers, outside the zone of aquaculture influence and should follow the same sampling recommendations provided here.

Preservation

12. Double wrap individual fish in heavy duty aluminum foil then double bag individual samples using appropriately sized, sterile bags ensuring that the sample identification label is included inside the outer bag and that bags are sealed with zap straps. Place clean cork stoppers over significant carapace spines prior to wrapping.
13. Store bagged fish on ice, in a closed cooler during collection.
14. Continue collecting dead or moribund fish observed in the vicinity of the aquaculture site using new gloves, new aluminum foil and a clean (see #1) wooden bat between each fish.
15. Upon completion, immediately transport all fish to a clean freezer, on-site, operating at -20 C or below.

Shipping

16. As soon as the fish are completely frozen and within one to two weeks[1] after collection, fish should be packed in coolers with dry ice, ice or cooler packs, etc. to ensure that they remain frozen, and shipped for analysis as described in *Preservation of Sample Integrity*.
17. Additionally, a sample of the applied chemotherapeutant should be provided to the analytical lab; the amount required, storage and shipping instructions will need to be obtained from the analytical lab.
18. Copies of the field note forms should be included with the shipment to the analytical lab. In advance of shipping, the lab should be informed on the details of the sample submission and on the analytical request.

[1] Degradation of target analytes such as emamectin benzoate are significantly slowed once frozen but it is recommended that samples be shipped as soon as possible and within 1-2 weeks to avoid unpredictable circumstances (such as power outages/equipment failures) that could compromise the samples.

19. Original field note forms and digital images should be stored in a secure cabinet until such time that they are submitted to the Minister.

Preservation of Sample Integrity

The preservation of sample integrity is crucial to prevent samples from being compromised and to ensure analytical accuracy. Aquaculture facilities are a source of chemotherapeutant contamination where treatment takes place or where therapeutants are stored. The following considerations will help to maintain sample integrity:

1. Samples, sampling supplies and equipment must be stored in a clean environment, away from therapeutants, to reduce contamination potential. Sealed, watertight containers are preferred for storing sampling supplies to help to prevent contamination.
2. All equipment must be cleaned with residue-free lab detergents and rinsed three times with distilled or tap water between sampling sessions and when there may have been exposure to therapeutants directly or via dust or water vapour.
3. During collection it is important to protect the integrity of the fish by ensuring fish do not have any skin, shell, or carapace lacerations and that no damage occurs to the outside of the fish. External lacerations provide entry points for contamination and can lead to fluid loss.
4. It is important that collection, storage and transport guidelines be followed. Low temperature storage is important to preserving the target analytes by preventing degradation. Fish should be kept in bags on ice during collection and subsequently frozen as soon as possible on-site at -20 C for storage. Once frozen, samples should remain frozen at constant temperature.
5. During transport to the lab, frozen fish should be packaged in coolers with ample supply of appropriate refrigerant (dry ice, ice or cooler packs, etc). The amount of dry ice required should reflect the sample weight and anticipated shipping time and calculated on advice from dry ice providers.
6. A completed chain of custody (COC) form should accompany every shipment and should be provided by the analytical lab on request prior to any shipment. COCs are updated when the samples change custodian. The aquaculture manager should keep a copy of the COC with the sampling documents and the lab will maintain a copy in their project files. This will ensure a complete historical record of custody and ensures that the samples analyzed were the same as the samples reported to have been taken at a particular time and place (e.g., the analytical results of a fish sample accurately represent the fish at the time and place of sampling).
7. Certain shipping guidelines must be followed when shipping with dry ice so shipping companies should be consulted for advice prior to shipping. Shipments should be initiated on a Monday to avoid weekend delays and longer than anticipated shipping times which could raise the shipping temperature and compromise the samples. Expedited shipping methods and tracking options are recommended for the transport of frozen fish.
8. Suggested requirements for preparing dry ice shipments (*Source UPS*):

   (i) Fill any empty space in your package with appropriate packing material to prevent product movement in transit.

   (ii) Avoid shipping temperature-sensitive products over the weekend.

(iii) Wrap the refrigerant in paper or another carton to slow the melting rate and prevent excess space when using dry ice.

(iv) Do not place the refrigerant at the bottom of the package because cold air will not circulate.

(v) Do not seal the inner insulated container when using dry ice. Venting is required to allow some carbon dioxide gas to escape the package.

Field Data Requirements

For the proper interpretation of analytical results it is important to collect all vital field data in a consistent and standardized method which requires the use of specific procedures, forms and labels. Data collection forms should include (but are not limited to) the specific information included below. Separate identification labels that accompany the samples during storage and shipment should be designed to correspond with the data collection forms. Indelible ink pens and field grade waterproof paper and labels would be recommended. Digital images of fish at capture would help to assess any morphological abnormalities and species identification at collection noting that the sample identification form should be included in the image. Video record of moribund fish behaviour might be of value for interpreting symptoms of exposure to certain chemicals.

Specific information requirements should include:

1. Aquaculture site and company name
2. Site manager and contact information
3. Sampler name and crew members
4. Depth and GPS coordinates of aquaculture site
5. Species, size, year class and biomass of fish on site at treatment time.
6. Chemotherapeutant product, method of application, application concentration, amount of product applied, and stage of application when affected fish were observed.
7. Date and time of collection
8. Time and location when unusual fish morbidity and mortality was first observed and name of observer(s)
9. Time and location (GPS coordinates) when fish were collected and names of collectors
10. Surface water temperature at time of sampling at farm site
11. Weather/Air Temperature
12. Collection method
13. Fish Identification Number
14. Photograph number
15. Length Measurements[2]

[2] Measure each finfish to determine fork length and total body length (mm). Total body length is measured from the anterior-most part of the fish to the tip of the longest caudal fin ray. Fork length is measured from the anterior-most part of the fish to the middle caudal fin rays. The body measurements for shellfish differ depending on the type; for bivalve molluscs height is measured from the umbo to the anterior (ventral) shell margin; for crabs, the total lateral width of the carapace is measured; for shrimp and prawns the

16. Total Weight (g)
17. Species, if identifiable
18. Condition: live or dead and if live, describe behaviour observed. Include video of moribund behaviour where possible.
19. Observed morphological abnormalities: such as evidence of tissue damage, growth abnormalities, lacerations, fin erosion, skin ulcers, neoplasms
20. Quantity and type of ectoparasites
21. Record of storage and transport conditions (i.e., time spent at ambient temperature, time stored on ice at collection and storage time at -20 C prior to shipping)

Suggested Sampling Equipment and Supplies

The table below summarizes the suggested supplies required for the collection of life stages of fish should there be an observed unusual fish morbidity or mortality event in the vicinity of an aquaculture facility.

carapace length is measured from tip of rostrum to the posterior margin of the carapace and total length is measured from the tip of the rostrum to the tip of the telson; and for lobsters, the length of the carapace is measured from the rear of the eye socket to the posterior margin of the carapace (USEPA 2000).

Table 1: Recommended equipment for collecting, preserving and transporting specimens for analysis.

Item Description	Number Required	On-Site Storage
Waterproof totes	as required	Indoor clean environment
1m x 0.3m clear sterile bag (approx.)	500	Waterproof Tote
50cm x 25cm clear sterile bag (approx.)	500	Waterproof Tote
15cm x 7cm clear sterile bag (approx.)	500	Waterproof Tote
Zap straps	500	Waterproof Tote
Waterproof field data forms	500	Waterproof Tote
Waterproof identification labels	500	Waterproof Tote
Indelible ink pens/pencils	10	Waterproof Tote
Clip Board	3	Waterproof Tote
Marine Dip Net: for large fish	1	Indoor clean environment
Marine Dip Net: for small fish	1	Indoor clean environment
Disposable Nitrile Gloves	1 Box (500)	Waterproof Tote
Outdoor GPS unit	1	Indoor clean environment
Digital Camera	1	Indoor clean environment
Measuring Tape	2	Waterproof Tote
Scale: field grade portable analytical balance.	1	Waterproof Tote
Flashlight	1	Waterproof Tote
Cooler	2	Indoor clean environment
Chest Freezer (-20 C)	1	Clean environment
Ice	10 bags	Freezer
Ice Packs	20	Freezer
Blunt instrument (wooden bat)	1	Waterproof Tote
Aluminum Foil	500m	Waterproof Tote

Analytical considerations when sampling for current-use chemotherapeutant active ingredients in fish tissues.

When collecting fish for the analysis of active ingredients of anti-sea lice chemotherapeutants in tissue samples, it is important to consider any target analyte limitations. Here, current-use products are defined to include registered products, those proposed for registration and those used under emergency release provisions in Canada to include SLICE®, Paramove® and Salmosan®. A brief description of limitations and considerations follows.

Pesticide: Paramove®

Paramove®'s active ingredient is hydrogen peroxide. Hydrogen peroxide is reactive, does not bioaccumulate in tissues (Schmidt et al. 2006, Burridge 2013) and undergoes accelerated degradation depending on several factors including enzymatic activity, temperature, light, and pH. Furthermore, hydrogen peroxide is a by-product of cellular metabolism and is naturally present in the environment at low levels (MassDEP 2010) presenting a consideration for any analysis and interpretation.

Paramove® limitation: Recognizing its susceptibility to rapid degradation, hydrogen peroxide it is not considered a suitable target analyte for detection in fish tissue.

Acute toxicity of hydrogen peroxide to Chinook and Atlantic Salmon has been determined through histological evidence from sections of gill samples which reveal extensive epithelial lifting and necrosis (Johnson et al. 1993). Other studies also show that there is a significant correlation between the level of exposure and the degree of gill damage (Kiemer et al. 1996). To assess acute effects in fish which may be attributable to hydrogen peroxide exposure, gill tissue should be sampled and preserved by methods suitable for subsequent histological analysis. However, gill histopathology can yield results that are not specific to a chemical so additional evidence may be needed, depending on the objectives.

Recommendation: Interpretation of histological damage in fish should be assessed as a better indicator of acute effects from a potential hydrogen peroxide exposure, but sampling demands live specimens and requires specialized tissue fixation procedures.

Pesticide: Salmosan®

Salmosan®'s active ingredient is azamethiphos, an organophosphate insecticide which acts by inhibition of acetylcholinesterase (AChE) activity. Studies have shown that absorption following topical treatment of azamethiphos in salmon is low, that there is no bioaccumulation and that elimination of total azamethiphos-related residues in salmon is rapid (Roth et al. 1993, EMEA 1999, Burridge 2013). Other studies have described degradation of azamethiphos in the lab during sample preparation (Pfenning et al. 1999). Under certain conditions, and with correct sample handing and analytical methods, it *may* be possible to measure azamethiphos in tissue immediately after exposure; however the factors described above limit confidence in negative results, especially if sample degradation may have occurred.

Salmosan® Limitation: Recognizing that fish rapidly metabolize azamethiphos, it is not considered a suitable target analyte for detection purposes in tissue.

Due to its rapid metabolism and degradation and the probability of low residue concentrations, another approach to assessing exposure to azamethiphos involves analysing for metabolites and/or biomarkers, but is outside the scope of the current request. Azamethiphos is a fairly powerful inhibitor of AChE in fish brain (e.g., Intorre et al. 2004) whose activity can be used to assess possible contamination by organophosphate pesticides (e.g., Kirby et al. 2000). However, the use of AChE inhibition as a biomarker requires the collection of live or moribund (not dead) samples, and specific tissue preservation steps need to be used (e.g., Jung et al. 2007).

Recommendation: Metabolites and biomarkers should be further assessed as alternate and complementary approaches to determine exposure to azamethiphos in fish.

Drug: SLICE®

The active ingredient of SLICE® is the avermectin derivative, emamectin benzoate (EB), which, upon ingestion, is absorbed from the gut and transported to other tissues. EB will accumulate at

low levels in farmed fish tissue during treatment but is eliminated over several months with an elimination half-life of about 8.5 - 11.5 days (Glover et al. 2010). EB has been measured in farmed salmon blood, mucus and muscle tissue during and after prescribed treatment (Sevatdal et al. 2005).

Of the three chemotherapeutants currently used in Canada, the in-feed treatment, SLICE®, has the most stable active ingredient (EB) that would allow detection in fish tissue.

Limitations and Considerations

There are a number of limitations and considerations which should be taken into account when implementing the advice provided in this response. These are:

Limitations

1. The sampling advice provided in this response encompasses finfish and shellfish (bivalves and crustaceans). The AAR definition of fish is that of the *Fisheries Act* (R.S.C., 1985, c. F-14), however, there are practical sampling limitations associated with recommending sampling protocols to cover the full definition. Thus, sampling advice will be provided excepting: marine mammals, parts of shellfish, crustaceans or marine animals, and the eggs, sperm, spawn, larvae, spat of shellfish, crustaceans and marine animals. Sampling of juvenile stages of fish will be limited to those size ranges that can be observed from an aquaculture facility.
2. The advice refers to anti-sea lice chemotherapeutants currently used in Canada: Salmosan®, Paramove® and SLICE®. Where new chemotherapeutant treatments are developed or where there are concerns with the use of unauthorized products, the AMS should be updated as necessary.

Considerations

1. The approach to 'monitoring' described here excludes systematic or repeated sampling (normally implied by the term "monitoring") and more accurately describes sampling in response to an event.
2. The advice provided only considers the sampling requirements for chemotherapeutant analysis. In certain instances where organisms death may be the result of factors other than chemotherapeutants or involves chemotherapeutants that do not result in detectable tissue residues, other health indicators or biomarkers of exposure may need to be measured and such parameters would require special sampling and preservation methods. It may also be important to measure environmental (e.g., dissolved oxygen, pH) and biological factors (e.g., harmful/toxic phytoplankton, pathological agents) that could cause mortality or increase fish sensitivity to chemotherapeutant exposure. In such circumstances, specific additional advice may be necessary and such determination would be up to the individual tasked with interpreting the information.
3. Any analysis of chemotherapeutants in suspect fish should be supported by appropriate biological measurements (age, sex, reproductive status, species or stock determinations) which would contribute to interpretation of analytical data. Native wild species would have to be distinguished from farmed species. This information could also help determine if the fish, for example, were discarded as by-catch from fishing operations, rather than being affected by aquaculture operations.
4. Where other aquacultures operations are nearby, it would be important to analyze tissue samples for a suite of pest control products.

5. The sampling advice could be applied when well boat treatment water is discharged at a distance from the aquaculture site, should this be required by the Regulations.
6. Sampling advice to avoid cross-contamination from the collection of potentially diseased fish may be of importance but has not been included here. The Canadian Food Inspection Agency (CFIA) has relevant expertise, and some authority to deal with biosecurity issues for producers, including aquaculture operators, and could be consulted on such matters.
7. This document is focused on the technical aspects of sampling. However, the specific circumstances of sampling will result in specific hazards to those undertaking the work. In providing this guidance, it is explicitly intended that those carrying out the sampling have been trained and have developed a Health and Safety Plan and carried out a site-specific safety risk assessment at the site in question.
8. Given the need for ensuring consistency, quality of samples, those carrying out the sampling should be trained appropriately.
9. There may be occasions where blood samples from moribund or live fish would be needed in addition to frozen whole fish for analytical purposes.
10. Within a 96-hour time frame following the deposit of drugs or pesticides, moribund or dead fish will likely be transported distances away from the site where the treatment occurred. The distance and direction will be dependent on local hydrography.
11. Well before any sampling a certain amount of preparatory work is recommended: (1) Target analytes should be defined (2) Standardized methods for extraction and analysis should be defined for all target analytes noting that requirements for limits of quantitation would likely be guided by known thresholds of effect. (3) Shipping companies and laboratories capable of performing the analyses should be identified and contacted to define analytical requirements, establish accounts, policies and procedures for sample submissions and to develop lines of communication.(4) Sampling procedures should be confirmed with the laboratory to ensure they satisfy the analytical requirements. (5) Minimum tissue requirements for chemical analysis need to be established with the lab so that if small fish are collected, the number of fish required to meet the tissue requirements can be calculated for composite design. Additional tissue may also be required when separate extractions are necessary for the analysis of multiple chemotherapeutants, lipid and moisture determinations and other analyses.
12. The objective of the sampling and analysis should be further refined, as this may affect recommendations for appropriate sampling strategy and sample size.
13. The most sensitive non-target species are crustaceans, which would not be readily observable at the surface.

Conclusions

- This Science Special Response was developed to provide guidance on the procedures to be followed when collecting dead and moribund wild fish carcasses for the chemical analyses of the active ingredients found in anti-sea lice chemotherapeutant products. The sampling protocol is to be followed in response to an observed and unexpected 'event' within 96 hours of application of an anti-sea lice chemotherapeutant at a finfish aquaculture site and does not constitute a monitoring protocol for the presence of chemotherapeutants in wild fish.
- To avoid contamination and to ensure a consistent and standardized approach, it is recommended that whole fish be collected, frozen and shipped to a lab for processing and analysis. Fish dissection and preparation of specific tissues for analysis should occur at an analytical lab, following standardized methods based on species, life stage and analytical requirements.
- Where possible, a representative sample size of around 50 fish each from affected and reference groups for each affected species should be collected. It is recommended that 15 fish from each group are analyzed to establish mean values and variances; at this point a decision can be made as to whether more analyses are required to refine the statistical analyses. Fish from the reference group should be chosen to be as similar as possible in terms of species and size to the affected fish.
- In advance of requiring fish sample collection by aquaculture facilities, preparatory work is required such that there are laboratories prepared to accept the samples, prepare the tissues for analyses and conduct the required analysis for the analytes of interest.
- Preservation of sample integrity is paramount to ensure analytical accuracy. Considerations for preserving sample integrity are provided and should be adhered to.
- When collecting fish for the analysis of active ingredients of anti-sea lice chemotherapeutants in tissue samples, it is important to consider any target analyte limitations. In this case, it is noted that hydrogen peroxide and azamethiphos, the active ingredients in Paramove® and Salmosan®, would be ineffectual targets for chemical monitoring.
- The advice provided in this response is qualified by a number of Considerations and Limitations and Recommendations that should be taken into account when considering this advice.
- Although the advice is specific to the suite of anti-sea lice chemotherapeutants currently used in Canada, the sample collection, preservation and transportation methods outlined could be followed when collecting dead and moribund fish without prior knowledge of the specific chemotherapeutant in question.

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Appendix 1

The number of samples required for analysis of emamectin residues following an accidental kill or similar event can be calculated, provided certain assumptions about possible doses, tissue concentrations and their variance are made.

Bright and Dionne (2005) report that the therapeutic dose of emamectin for Atlantic Salmon is 0.05 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} in feed for 7 days (it is assumed that this refers to dose/kg fish wt.). This corresponds to a total dose over 7 days of 0.35 mg $\cdot$ kg^{-1}. Glover et al. (2010) treated Atlantic Salmon (approx. wt. 50 g) intraperitoneally (IP) with a single dose of 0.40 mg $\cdot$ kg^{-1} of emamectin and after 14 days measured muscle tissue concentrations of 0.167 ± 0.044 mg $\cdot$ kg^{-1} tissue (mean ± SD); i.e., the SD was approx. 25% of the mean residue concentration. (This mean SD ratio was fairly consistent for emamectin doses ranging from 0.1 to 0.8 mg $\cdot$ kg^{-1}.). Although the data of Glover et al. (2010) refer to IP injection, it is assumed for the purposes of the following calculation that emamectin uptake from food would be in the same range as that absorbed following IP injection.

As discussed in the main text, it is recommended that following an event, samples of affected fish would be taken from both the putatively contaminated population, and from a "reference" population which was known not to have been exposed to emamectin. The statistical basis for calculating the number of samples *from both affected and reference fish* is described in Rosner (2010) Eqn. 8.26:

$$n = [(\sigma_1^2 + \sigma_2^2)\,(z_{(1-\alpha/2)} + z_{(1-\beta)})^2]/(\mu_1 - \mu_2)^2$$

For an arbitrarily chosen statistical power of 0.8, and a type I error frequency of 0.05, the second term of this equation is a constant value of

$$(1.96 \pm 0.84)^2 = 7.84$$

So the equation becomes:

$$n = [7.84 \times (\sigma_1^2 + \sigma_2^2)]/(\mu_1 - \mu_2)^2$$

where *n* is number of samples required for each group;

$\sigma_1^2$and σ_2^2 are the variances of populations 1 and 2, respectively;
μ_1 and μ_2 are the mean values of populations 1 and 2 respectively;
values of z are taken from statistical tables widely available.

In practice, values of σ and of μ are rarely available, and *sample* SD and mean values must be used as an approximation.

In other words – and not surprisingly – the number of samples required to reliably detect a statistically significant difference between two means depends on the variance of the data and (inversely) on the difference between the means.

Using the data from Glover et al. (2010) and assuming the investigator wants to detect a 5-fold difference in residue concentrations between reference fish and those exposed to a therapeutic dose of emamectin (i.e., 0.167 ± 0.044 mg $\cdot$ kg^{-1} in exposed fish, and 0.033 ± 0.008 mg $\cdot$ kg^{-1} in reference fish), with a statistical power of 0.8 and a type I error rate of 0.05, *n* turns out (rather surprisingly) to be 1 fish from each group (Fig. 1). However, this reflects the very "tight" SD in the data derived from a controlled laboratory exposure, and furthermore, does not allow any estimate of variance in the data. Although the conclusion may be valid statistically, it is not useful operationally. Variance in less well-controlled field situations is likely to be much higher, and Fig. 1 also shows the sample numbers required *from each group* for increasing variances. Experiments with organic contaminants with K_{ow} (octanol/water partition coefficient) similar to that of emamectin, and absorbed similarly through food, shows SD in field samples are often

50% or more of mean values (e.g., Ikonomou et al. 2011). For the purpose of this document, it is recommended that a variance of 100% of the mean value be assumed. This requires a minimum sample size of 13 individuals from each group, which it is recommended should be rounded up to 15.

If, instead of seeking a 5-fold difference between means of affected and reference groups, the investigator seeks a 4-, 3- or 2-fold difference, the number of samples from each group is respectively 15, 19 and 40.

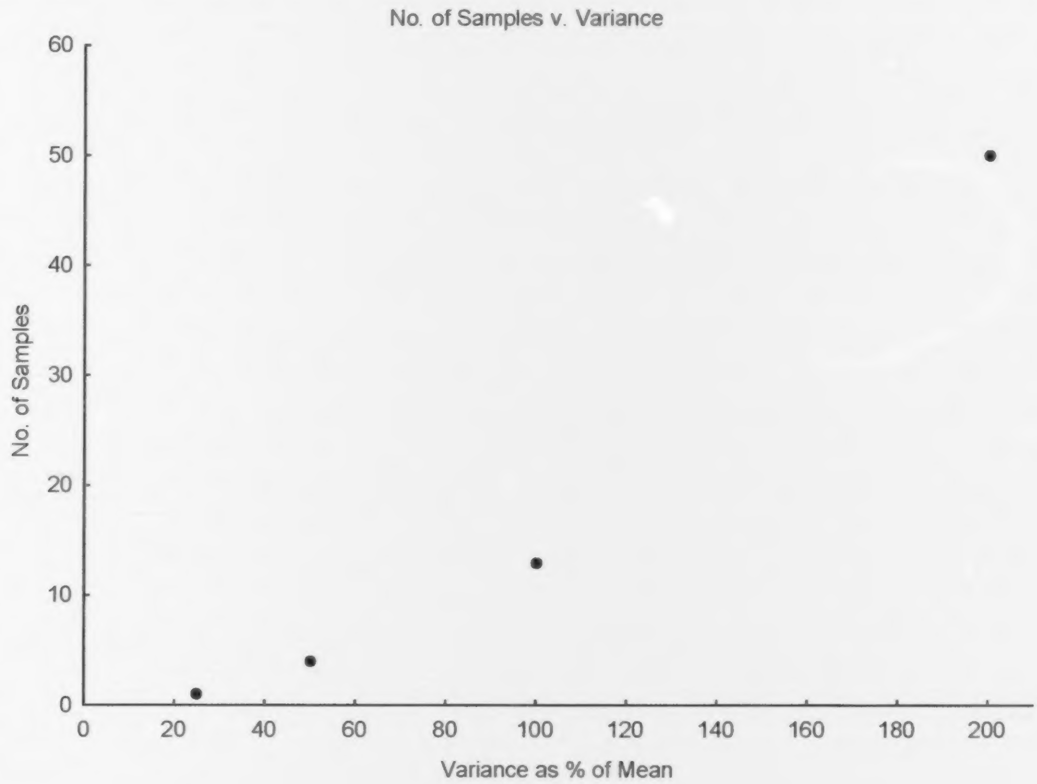

Figure 1. Number of samples required to detect a 5-fold difference between residue concentrations in affected and reference fish at different levels of variance. Calculations assume power of 0.8, type I error rate of 0.05, and residue concentrations quoted by Glover et al. (2010) as described in text.

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